Delayed replication timing leads to delayed mitotic chromosome condensation and chromosomal instability of chromosome translocations

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Chromosomal rearrangements are found in virtually all types of human cancers. We show that certain chromosome translocations display a delay in mitotic chromosome condensation that is associated with a delay in the mitosis-specific phosphorylation of histone H3. This delay in mitotic condensation is preceded by a delay in both the initiation as well as the completion of chromosome replication. In addition, chromosomes with this phenotype participate in numerous secondary translocations and rearrangements. Chromosomes with this phenotype were detected in five of seven tumor-derived cell lines and in five of thirteen primary tumor samples. These data suggest that certain chromosomal rearrangements found in tumor cells cause a significant delay in replication timing of the entire chromosome that subsequently results in delayed mitotic chromosome condensation and ultimately in chromosomal instability.

Cancer cells differ from their normal cellular counterparts in many important characteristics, including loss of differentiation, increased genomic instability, and decreased drug sensitivity. Not surprisingly, genetic alterations occur in most, if not all cancer cells, and are thought to lie at the heart of these phenotypic alterations. Furthermore, molecular analysis of individual tumors often reveals multiple genetic changes, including chromosomal translocations, deletions, insertions, gene amplifications, and point mutations. Recent surveys have identified more than 2,000 recurrent chromosomal aberrations among different neoplastic disorders (1, 2). However, the molecular and phenotypic alterations that are associated with the majority of these chromosomal changes remain undefined. The results described in this report characterize a new type of chromosomal abnormality that occurs with certain chromosome rearrangements, and is associated with abnormal chromosome replication timing, abnormal mitotic chromosome condensation, and considerable chromosomal instability.

Methods

Cells. C2C12, CRL-5845, CRL-5824, HTB-81, HTB-118, WERI-RB1, and HELA cells were from the American Type Culture Collection. RH30 cells were provided by P. Houghton (St. Jude Children's Hospital, Memphis, TN). All cell lines were grown in DMEM supplemented with 10% FBS (HyClone). CRL-5845 and RH30 cells were stably transfected with pRSVNEO by electroporation (300 volts, 950 μF in PBS; Bio-Rad), and $\approx$2,000 clones were pooled and expanded for use as donors in microcell fusions.

Microcell Mediated Chromosome Transfer. Donor cells were micronucleated by adding 10.0 μg of colcemid per ml in DMEM plus 15% calf serum for 48 h. The micronucleate cell populations were enucleated by centrifugation in the presence of 5 μg of cytochalasin B (Sigma) per ml, and the isolated microcells were fused to C2C12 recipients as described (3, 4). Microcell hybrids were isolated by using cloning cylinders after 3–4 weeks of selection in medium containing 500 μg of Geneticin (GIBCO) per ml.

Fluorescent *in Situ* Hybridization. Chromosome preparations from primary tumors were harvested in the absence of colcemid treatment as described (5). Slides of chromosomally normal metaphase spreads were obtained from peripheral blood (6), and slides from cell lines were prepared either in the presence or absence of colcemid (0.06 μg/ml) as described (4). DNA probes were nick-translated by using standard protocols to incorporate biotin-11-dUTP or digoxigenin-dUTP. Hybridizations were carried out on slides at 37°C for 16 h. Final probe concentrations varied from 40–60 ng/μl. Signal detection was carried out according to Trask and Pinkel (7). Chromosome-specific painting probes were used according to the manufacturer's recommendations (Vysis, Downers Grove, IL). Amplification of digoxygenated probes used alternating incubations of slides with FITC-tagged sheep antibodies made in rabbit and FITC-tagged rabbit antibodies made in sheep (Boehringer Mannheim). Slides were stained with propidium iodide (0.3 μg/ml), coverslipped, and viewed under UV fluorescence with FITC filters (Zeiss).

Replication Timing and Immunofluorescence. Cells were exposed to a pulse of 20 μg/ml of BUDR (Sigma) for 15 min, washed with PBS, and chased for various times in media containing 0.2 mM thymidine. Mitotic cells were harvested in the absence of colcemid at hourly intervals for up to 12 h. The cells were treated with 75 mM KCl for 15 min at 37°C, fixed in 3:1 methanol:acetic acid and dropped on wet slides. The chromosomes were denatured in 70% formamide in 2× SSC (1× SSC is 150 mM NaCl/15 mM Na-citrate) at 70°C for 3 min. Incorporated BUDR was detected by using an FITC-labeled anti-BUDR antibody (Becton Dickinson). Phosphorylated histone H3 was detected by using an antibody against phosphorylated serine 10 of H3 (Upstate Biotechnology, Lake Placid, NY). Slides were stained with propidium iodide (0.3 μg/ml), coverslipped, and viewed under UV fluorescence (Zeiss).

Results

Chromosome 3q Translocations Are Undercondensed During Mitosis. We have generated a series of microcell hybrid panels that contain different tumor-derived chromosomes. Chromosomes from the rhabdomysoarcoma cell line RH30 and the small-cell lung carcinoma (SCLC) cell line CRL-5845 were tagged by transfection and random integration of a neo^r gene, and transferred individually into C2C12 cells by means of microcell fusion. C2C12 cells are a murine myoblast cell line that we have used as

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Abbreviations: DMC, delay of mitotic chromosome condensation; DRT, delay of chromosome replication timing; FISH, fluorescence *in situ* hybridization.

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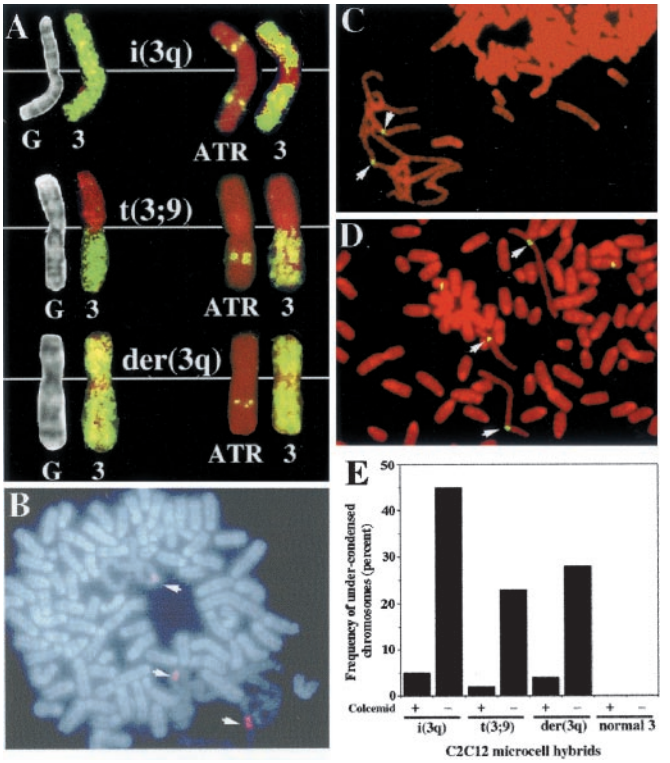

Fig. 1. Chromosome 3q translocations. (*A*) Karyotypic and FISH analysis of the i(3q), the t(3q;9q), and the der(3q) chromosomes. The i(3q) was isolated in microcell hybrids from the rhabdomyosarcoma cell line RH30 and the t(3;9) and der(3q) chromosomes were from the SCLC line CRL-5845. Mitotic spreads were analyzed by G-banding (G) followed by FISH with a chromosome 3 paint (3); or by FISH with an ATR probe followed by FISH with a chromosome 3 paint (3). Mitotic chromosome spreads from cells containing the i(3q) (*B*), the der(3q) (*C*), or the t(3q;9q) (*D*). The chromosome 3 translocations were identified by using either a chromosome 3-specific alpha satellite probe (*B*) or a human-specific pancentromeric probe (*C* and *D*). Arrows indicate the chromosome 3 translocations. The DNA was visualized by staining with either DAPI (*B*) or propidium iodide (*A*, *C*, and *D*). (*E*) The frequency of undercondensed chromosomes was determined in C2C12 microcell hybrids containing the i(3q), the t(3;9), the der(3q), or a nonrearranged chromosome 3. Metaphase spreads were prepared in the presence or absence of a 30-min colcemid treatment. A minimum of 100 spreads from the translocation lines, and 1,000 spreads from the nonrearranged chromosome 3 line were scored for undercondensed chromosomes.

chromosome recipients in the characterization of chromosomal alterations that induce abnormal tumor cell phenotypes (8–10). During karyotypic analysis of hybrids containing three different translocations, an i(3q)(q10), a t(3;9)(q10;q10), and a der(3q)(q10;?) (Fig. 1*A*), we noticed that the translocation chromosomes appeared to be undercondensed in a fraction (less than 10%) of the metaphase spreads (Fig. 1 *B–D*). In addition, we found that only the 3q translocations, and not the C2C12 mouse chromosomes, displayed this undercondensed phenotype (Fig. 1 *B–D*).

Because these undercondensed chromosomes appear to be in the early stages of mitosis, whereas the other chromosomes appear to be in mid to late metaphase, we hypothesized that the relatively low frequency of undercondensed chromosomes in these mitotic spreads was due to the fact that our chromosome preparation procedure is designed to analyze chromosomes during mid to late metaphase. Recent studies have indicated that colcemid, in addition to causing a metaphase delay, causes a delay in the G_2 phase of the cell cycle, resulting in a dramatic reduction in the frequency of cells in prophase and prometaphase (11). Therefore, because our previous mitotic preparation procedure included colcemid pretreatment, we assayed mitotic spreads prepared in the absence of colcemid. To quantify the frequency of undercondensed chromosomes, we classified a chromosome as undercondensed if it displayed at least two of the following characteristics: it was at least twice as long as any other chromosome within the same spread (Fig. 1 *B–D*), it was less than half as wide as any other chromosome within the same spread (Fig. 1*D*), and/or it contained a bend of greater than 180° (Fig. 1 *B* and *C*). Mitotic spreads prepared from cells containing the i(3q), the t(3:9), or the der(3q) in the absence of colcemid showed a dramatic increase in the fraction of cells with undercondensed chromosomes (Fig. 1*E*). In contrast, analysis of mitotic spreads from a C2C12 microcell hybrid containing a nonrearranged human chromosome 3 showed an undetectable frequency of undercondensed chromosomes, either in the presence or absence of colcemid, with over 1,000 mitotic spreads analyzed (Fig. 1*E*). We conclude that these three rearranged chromosomes display this undercondensed phenotype in a significant fraction of the mitotic events in these hybrid cell populations, and that colcemid pretreatment interferes with our ability to detect this phenotype.

Delay of Histone H3 Phosphorylation During Mitosis. Proper chromosome condensation during mitosis is associated with phosphorylation of histone H3 (12). Furthermore, there is a precise spatial and temporal correlation between H3 phosphorylation and the initial stages of chromatin condensation during mitosis (13). Therefore, to determine whether the initial stages of chromosome condensation were altered on the chromosome 3 translocations, we analyzed phosphorylation of histone H3 on undercondensed chromosomes by using an antibody specific for phosphorylation on serine 10 of H3. Immunofluorescence analysis indicated that phosphorylation of H3 was not detected on the undercondensed i(3q)s (Fig. 2 *A* and *B*). In contrast, phosphorylation of H3 was detected on the mouse chromosomes in the same mitotic spreads. However, phosphorylation of H3 was detected on the i(3q)s when they appeared fully condensed either in the presence (data not shown) or absence (Fig. 2*F*) of colcemid. These results indicate that the undercondensed state of these chromosomes is associated with a delay in the mitosis-specific phosphorylation of histone H3. Therefore, we will use the term *d*elay of *m*itotic chromosome *c*ondensation (DMC) to describe this undercondensed phenotype.

DMC Occurs in Tumor-Derived Cell Lines. We next tested whether omitting colcemid pretreatment would allow us to detect chromosomes with DMC in the parental tumor cell lines. Chromosomes with DMC were detected in greater than 30% of the mitotic spreads prepared in the absence of colcemid from either RH30 or CRL-5845 cells (Fig. 3*A*). In contrast, chromosomes with DMC were not detected in mitotic spreads prepared with colcemid from either cell line. In addition, it should be noted that the identity of the chromosomes with DMC in these tumor cell lines remains unknown, primarily because the undercondensed state of these chromosomes prohibits proper chromosome banding. However, fluorescence *in situ* hybridization (FISH) analysis, using a chromosome 3-specific paint probe, indicated that 10 of 15 chromosomes with DMC in the RH30 cells were positive for chromosome 3, indicating that at least two different chromosomes display the DMC phenotype in the RH30 cell line, and that one of them is derived from chromosome 3. A representative mitotic spread shows that the chromosome 3 probe hybridized to a chromosome with DMC and to a chromosome with normal condensation in the same mitotic spread (Fig. 3*B*). G-banding indicated that the chromosome with normal condensation was a nonrearranged chromosome 3 (Fig. 3*C*). These observations suggest that the chromosome with DMC that

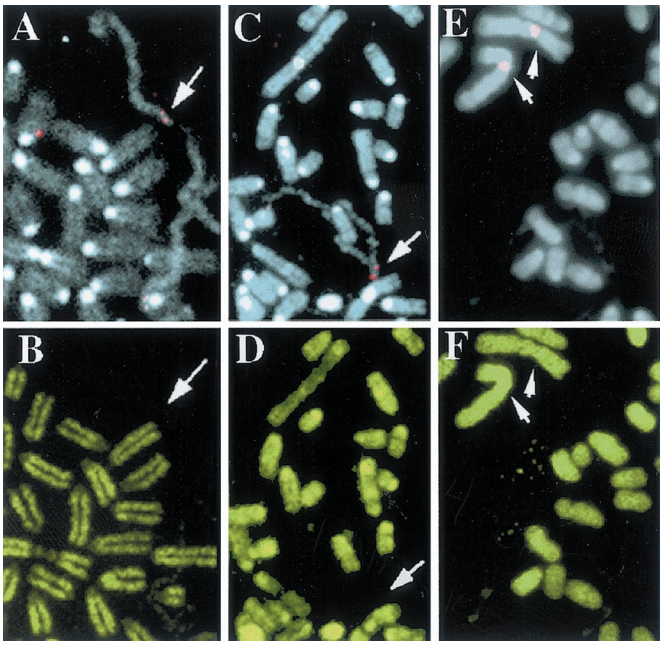

Fig. 2. Delay of mitotic phosphorylation of histone H3. Mitotic chromosome spreads containing the i(3q) were stained with an antibody specific for phosphorylation of serine 10 of histone H3 (*B*, *D*, and *F*). The i(3q)s in each spread were identified by using FISH with a chromosome 3 alpha satellite probe, and the chromosomes were visualized by using DAPI (*A*, *C*, and *E*). Two mitotic spreads with undercondensed chromosomes (*A*–*D*) and a mitotic spread with two i(3q) chromosomes with apparently normal condensation and phospho-H3 staining (*E* and *F*) are shown.

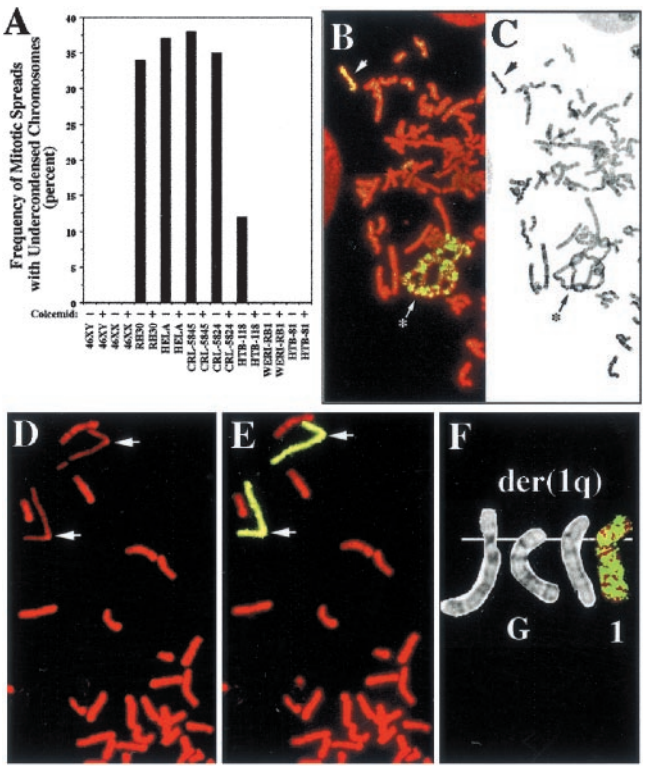

Fig. 3. Delay of mitotic chromosome condensation in tumor cell lines. (*A*) Mitotic chromosome spreads were scored for the presence of chromosomes with DMC in tumor derived cells. Chromosomes with DMC were scored in mitotic spreads harvested either in the presence (+) or absence (−) of colcemid from the blood of two normal individuals (46XY and 46XX) and from the tumor-derived cell lines RH30, CRL-5845, CRL-5824, HTB-118, WERI-RB1, and HTB-81. Following propidium iodine staining, a minimum of 100 mitotic spreads was scored for each sample. Sequential G-banding (*C*) and FISH (*B*) with a chromosome 3 paint on a mitotic spread from RH30 cells prepared in the absence of colcemid. This probe hybridized to a nonrearranged chromosome 3 (arrow), and to a chromosome with DMC (* and arrow). (*D* and *E*) Mitotic chromosome spreads showing two copies of the der(1q) (*D*) that hybridize to a chromosome 1 paint (*E*). (*F*) Mitotic spreads were analyzed sequentially by G-banding (G) followed by FISH with a chromosome 1 paint (1). The DNA was visualized in the FISH analyses by staining with propidium iodide.

hybridized to the chromosome 3 paint is likely to be the i(3q). A similar FISH analysis on mitotic spreads from the CRL-5845 cell line indicated that chromosomes with DMC also hybridized to the chromosome 3 probe, suggesting that the t(3;9) and/or the der(3q) also display DMC in the parental tumor cell line (see Table 2, which is published as supporting information on the PNAS web site, www.pnas.org). These results indicate that chromosomes with DMC can be detected in the parental tumor cell lines, but only in the absence of colcemid.

To determine whether chromosomes with DMC are common in other cell lines, we analyzed mitotic spreads from five additional tumor-derived cell lines. We detected chromosomes with DMC in greater than 30% of the mitotic spreads from the cervical carcinoma cell line HELA and a second SCLC cell line CRL-5824, and in greater than 10% of the mitotic spreads from the vulva carcinoma cell line HTB-118 (Fig. 3*A*). Again, the ability to detect chromosomes with DMC depended on omitting colcemid pretreatment. In contrast, chromosomes with DMC were not detected in mitotic spreads from the retinoblastoma cell line WERI-RB1 or from the prostate carcinoma cell line HTB-81. Thus, we detected DMC in five of seven tumor derived cell lines, suggesting that DMC is common in tumor-derived cell lines. Furthermore, the WERI-RB1 cells contain an isochromosome 6p in greater than 75% of mitotic spreads, indicating that not all isochromosomes display the DMC phenotype. In addition, chromosomes with DMC were not detected in mitotic spreads harvested from the blood of two normal individuals (Fig. 3*A*).

To begin to characterize the chromosomes with DMC that did not hybridize to the chromosome 3 probe, we analyzed mitotic spreads from CRL-5845 and HELA using FISH and chromosome painting probes for chromosomes 1, 3, 12, and 17. We chose chromosomes 1, 12, and 17 because isochromosomes and whole-arm translocations involving these chromosomes are common in many tumor cell types (1). This analysis indicated that both HELA and CRL-5845 cells contain chromosomes with DMC that hybridize to the chromosome 12 probe. Furthermore, CRL-5845 cells contain chromosomes with DMC that hybridize to the chromosome 1 probe. In contrast, we have not detected hybridization of the chromosome 17 probe to chromosomes with DMC in any of the cell lines tested (Table 2).

The detailed karyotypic analysis of the chromosome 3 alterations described above was facilitated by the isolation of these chromosomes in microcell hybrids. Therefore, to characterize the chromosome with DMC that hybridized to the chromosome 1 probe in the CRL-5845 cells, we screened the CRL-5845 microcell hybrid panel for retention of DNA markers located on human chromosome 1. This analysis identified six independent clones, which were positive for human chromosome 1. FISH analysis with the chromosome 1 paint indicated that one clone, C2(5845n)-9, retained a single human chromosome, and that this human chromosome displayed DMC (Fig. 3 *D* and *E*). The other five clones did not display DMC and consequently were not analyzed further. Karyotypic analysis indicated that the human chromosome present in C2(5845n)-9 was a derivative of chromosome 1q, der(1q)(q10;?) (Fig. 3*F*). This chromosome retains an apparently normal q arm and a highly rearranged short arm.

Table 1. Delay of mitotic chromosome condensation in primary tumors

Sample	Diagnosis	Total # of spreads	Spreads with DMC
2371	Carcinoma, cervical	20	1
2271	Carcinoma, papillary	35	1
2285	Carcinoma, squamous cell	40	2
2286	Carcinoma, cervical	20	0
2300	Plasmacytoma	35	1
2301	Leiomyoma	50	0
2309	Adenoma	40	0
2317	Carcinoma, clear cell	5	0
2320	Carcinoma	31	1
2321	Carcinoma	50	0
2191	Carcinoma, transitional cell	10	0
2375	Osteosarcoma	5	0
2232	Seminoma	15	0

Primary tumor samples were obtained directly from patients undergoing surgery or biopsy. Mitotic spreads were prepared in the absence of colcemid and scored for the presence of chromosomes with DMC. The total number of mitotic spreads analyzed and the number of spreads with a chromosome with DMC is indicated.

These results indicate that at least one other translocation chromosome involving deletion and/or rearrangement of its short arm displays the DMC phenotype.

Delay of Mitotic Chromosome Condensation in Primary Tumors. To determine whether chromosomes with DMC could be detected in the mitotic spreads of primary tumors, we analyzed mitotic spreads harvested directly from primary tumor samples prepared in the absence of colcemid. From a total of 19 different tumor samples, we were able to score metaphase spreads from 13 different tumors. Chromosomes with DMC were detected, albeit at a low frequency, in the metaphase spreads from five different primary tumors (Table 1). This analysis was hampered by the fact that mitotic spreads are rare in primary tumors harvested in the absence of colcemid. In addition, the chromosomes present in the majority of the mitotic spreads from these direct harvests had a highly condensed appearance, suggesting that the mitotic cells in these preparations were primarily in late metaphase. Therefore, because human cells arrested in late metaphase with colcemid have an undetectable frequency of chromosomes with DMC, we believe that this analysis results in an underestimate of the frequency of chromosomes with DMC in primary tumors. Regardless, chromosomes with DMC were detected in the mitotic spreads of primary tumors.

Delay of Chromosome Replication Timing. Mammalian chromosomes synthesize DNA in a segmental and highly coordinated fashion during each S phase, with euchromatin replicating early and heterochromatin replicating late (14). We determined whether replication timing was altered on chromosomes with DMC. For this analysis, we combined BUDR incorporation with mitotic chromosome analysis of metaphase spreads prepared in the absence of colcemid. The design of these experiments is illustrated in Fig. 4*A*. Examination of mitotic spreads harvested for early replication indicated that the highly condensed chromosomes incorporated BUDR along their length and that chromosomes with DMC did not incorporate BUDR (Fig. 4*B*). Furthermore, analysis of mitotic spreads harvested for late replication indicated that chromosomes with DMC incorporated BUDR along their length at a time when the fully condensed chromosomes were incorporating BUDR into their centromeric regions (Fig. 4 *C* and *D*). Because centromeric regions are primarily late replicating (15), we conclude that the chromosomes with DMC replicate extremely late. Furthermore, we have detected chromosomes with DMC that display a banded pattern of BUDR incorporation (Fig. 4*D*), indicating that chromosomes with DMC retain regions of DNA that replicate before other

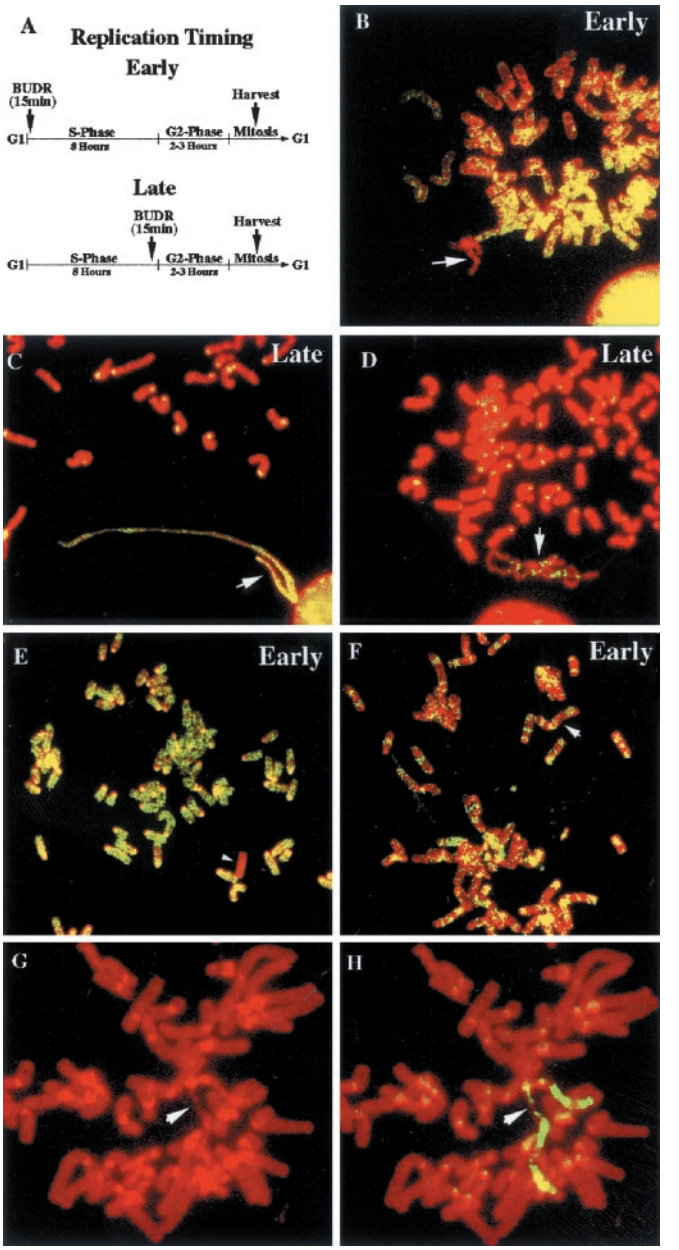

Fig. 4. Delay of replication timing on chromosomes with DMC. (*A*) General scheme for replication timing experiments, showing the time of the BUDR pulse and the time of mitotic cell harvest for early and late replication. Incorporated BUDR was detected by using an FITC labeled anti-BUDR antibody, and the DNA was detected by using PI. (*B*) A representative mitotic spread from cells containing the i(3q) harvested for early replication. The arrows mark the chromosomes with DMC (*B–D*). (*C*) A representative mitotic spread from cells containing the i(3q) harvested for late replication. (*D*) A mitotic spread from cells containing the i(3q) harvested for late replication showing a banded pattern of BUDR incorporation. (*E*) A representative mitotic spread from the parental C2C12 cells harvested for early replication. The arrow marks a late replicating X chromosome. (*F*) A representative mitotic spread from C2(3n)-1 cells harvested for early replication. The arrow marks the nonrearranged human chromosome 3. (*G* and *H*) A representative mitotic spread from CRL-5845 harvested for late replication (*G* shows staining with PI only, and *H* shows PI plus BUDR).

regions. We estimate that the chromosomes with DMC are delayed in initiation of DNA synthesis by at least 3 h, and that completion of DNA synthesis is also delayed by 2–3 h. These observations indicate that chromosomes with DMC show a delay in their initiation as well as completion of DNA replication. Therefore, we will use the term *d*elay of chromosome *r*eplication *t*iming (DRT) to describe this phenotype.

A series of control experiments were carried out to show that our replication timing procedure detects both early and late replication of chromosomes in the C2C12 cell background. First, analysis of parental C2C12 mitotic spreads harvested for early replication indicated that BUDR incorporation was detected in all of the chromosomes except for one of the X chromosomes (Fig. 4*E*), indicating that our replication timing procedure can detect late replication of an inactive X. Second, analysis of a nonrearranged chromosome 3, in a C2C12 microcell hybrid, indicated that normal chromosome 3 retains both early (Fig. 4*F*) and late (data not shown) replicating regions, consistent with the known replication timing pattern for this chromosome (14). Finally, we found that a derivative chromosome 14, containing a homogeneously staining region (HSR), also present in a C2C12 microcell hybrid, is primarily early replicating (see Fig. 7, which is published as supporting information on the PNAS web site, www.pnas.org). These results are consistent with previous observations that the DNA within HSRs are early replicating (16). Therefore, our replication timing procedure allows us to monitor both early and late replication timing, and indicates that a nonrearranged human chromosome 3 displays normal replication timing when introduced into C2C12 cells.

Furthermore, we determined whether chromosomes with DMC in the parental tumor cells also displayed DRT. We found that the chromosomes with DMC in CRL-5845 cells were late replicating. Fig. 4 *G* and *H* shows a mitotic spread harvested for late replication, and indicates that a chromosome with DMC incorporated BUDR at a time when the other chromosomes were incorporating BUDR into their centromeres. This analysis indicates that DRT occurs on chromosomes with DMC in the parental cell line CRL-5845.

Chromosomal Instability of the 3q Translocations. During routine culture of the microcell hybrids containing the chromosome 3 translocations, we noticed that the frequency of chromosomes that displayed DMC decreased with increasing passage, dropping to near undetectable levels between passages 15–20. Karyotypic analysis of late passage cultures indicated that the der(3q) was no longer present in its original form. FISH analysis, using a chromosome 3 painting probe, indicated that the chromosome 3 DNA was still present in these cells, but was found as numerous translocations to different mouse chromosomes (Fig. 5*A*). Similar rearrangements were observed in late passage cultures of cells containing the i(3q), the t(3;9), and the der(1q) chromosomes (data not shown). Furthermore, translocation intermediates involving the translocation chromosomes can be detected in early passage (5–10) cultures. Fig. 5*B* shows FISH analysis, using the chromosome 3 paint probe, on a mitotic spread containing a radial chromosome that involves the i(3q) and a mouse chromosome. A similar analysis of a C2C12 microcell hybrid with a nonrearranged chromosome 3 indicated that normal chromosome 3 remained intact and did not undergo similar translocations with increasing passage (data not shown). These observations indicate that chromosomes with DMC/DRT are unstable and participate in numerous secondary chromosomal rearrangements. In addition, once translocated onto mouse chromosomes the chromosome fragments no longer displayed DMC. Therefore, all of the experiments in this report were conducted with early passage (<10) cultures of the microcell hybrids that carry these rearranged chromosomes.

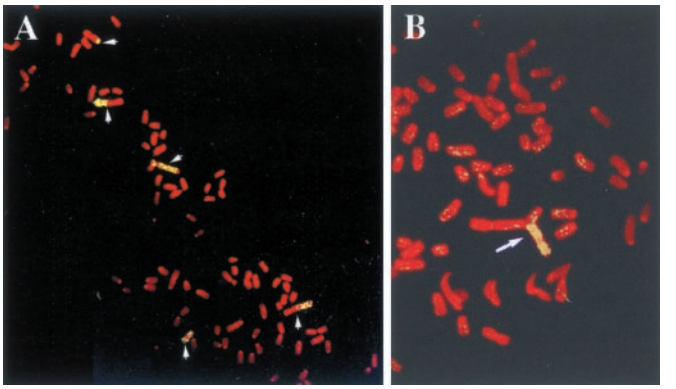

Fig. 5. Chromosomal instability. Numerous secondary alterations of the chromosome 3 translocations were observed in late passage (15–20) cultures. Mitotic spreads were subjected to FISH analysis with the chromosome 3 painting probe. (*A*) A representative mitotic spread from late passage cultures of cells with the der(3q). The arrows mark numerous translocations involving human chromosome 3 and the C2C12 mouse chromosomes. (*B*) A mitotic spread from cells with the i(3q) with a radial chromosome involving the i(3q) and a mouse chromosome.

Discussion

In this report, we describe a previously unknown chromosomal abnormality that occurs on a subset of chromosome rearrangements. We show that two different whole-arm translocations, and two different deletion/rearrangement chromosomes display a significant DMC that is associated with a delay in the mitosis-specific phosphorylation of histone H3. Chromosomes with DMC were detected in five of seven tumor-derived cell lines, and were detected at a low frequency in primary tumors. Furthermore, chromosomes with DMC are DRT of the entire chromo-

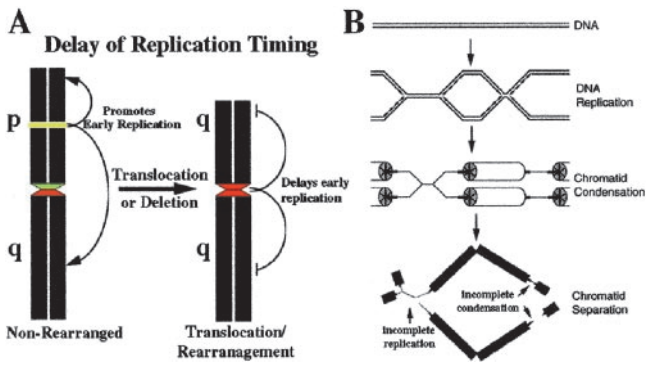

Fig. 6. Models for DRT and chromosomal instability. (*A*) We are considering two possible mechanisms that could result in DRT/DMC. First, because all of the translocations with DRT/DMC involve deletion and/or rearrangements of one arm of the affected chromosome, it is possible that deletion or mutation of a cis element (shown in yellow) that normally establishes early replication timing has occurred. Deletion of this element would then result in delayed replication of the entire chromosome. Second, because all of the translocations with DRT/DMC involve translocations or rearrangements in or near the centromeres of the affected chromosomes, it is possible that this type of chromosomal rearrangement actively interferes with normal chromosome replication timing by some unknown mechanism. (*B*) Schematic diagram of: DNA, DNA replication, chromatid condensation, and chromatid separation of a chromosome with DRT/DMC. We propose that delayed replication results in incomplete replication and/or incomplete mitotic chromosome condensation that persists into mitosis. Consequently, during chromatid separation either unreplicated DNA causes a break, or incomplete condensation results in a ''weak'' spot that causes a break during chromatid separation at anaphase. This model was adapted from the ''late-replicating DNA'' model for fragile site expression (21).

some, and participate in numerous secondary translocations and rearrangements.

Chromosome Condensation. The faithful segregation of genetic material during each cell division requires orchestrated changes of chromosome structure during mitosis. In early mitosis, dramatic structural changes occur to produce metaphase chromosomes, each consisting of a pair of condensed sister chromatids. Phosphorylation of histone H3 occurs at the onset of mitosis, and is required for proper chromosome condensation and segregation (12). Mitosis-specific phosphorylation of H3 initiates within the pericentromeric heterochromatin, spreads along the condensing chromatin fiber, and is completed just before the formation of prophase chromosomes (13). We show that a subset of chromosome rearrangements display a dramatic delay in phosphorylation of H3 during mitotic chromosome condensation. However, it is likely that these chromosomes eventually become fully or at least partially condensed, because we do detect phosphorylation of H3 on these chromosomes when they appear condensed during metaphase. These observations indicate that these chromosomes have a defect in the timing of mitotic chromosome condensation. However, as discussed below, it is likely that DMC occurs as a result of delayed replication.

Chromosome Replication Timing. Mammalian chromosomes synthesize DNA in a highly coordinated fashion during each S phase, with euchromatin replicating early and heterochromatin replicating late (14). We found that early DNA replication on chromosomes with DMC was delayed by at least 3 h. Similarly, we found that chromosomes with DMC were still replicating 2–3 h after the other chromosomes had completed replication. These observations indicate that the chromosomes with DMC are delayed in both the initiation and the completion of DNA replication. However, chromosomes with DMC do display a banded pattern of DNA synthesis, indicating that replication of some regions of these chromosomes occurs before other regions. This observation suggests that chromosomes with DMC retain both "early" and "late" replication, but that the early regions of these chromosomes do not replicate until the normal chromosomes are replicating their late regions. This interpretation suggests that each chromosome regulates its own replication timing independently, and that the replication pattern of individual chromosomes is controlled in a sequential manner so that the late replicating regions can only replicate after the early regions.

One question that remains unanswered concerning chromosomes with DRT/DMC is: How do chromosome translocations or rearrangements cause a persistent delay in replication timing? We are considering two possibilities (Fig. 6*A*). First, because all of the translocations with DRT/DMC involve deletion and/or rearrangements of one arm of the affected chromosome, it is possible that deletion or mutation of a cis element that normally establishes early replication timing has occurred. Deletion of this element would then result in delayed replication of the entire chromosome. Second, because all of the chromosomes with DRT/DMC involve translocations or rearrangements in or near their centromeric regions, it is possible that this type of rearrangement actively interferes with normal chromosome replication timing by some unknown mechanism. We cannot distinguish between these possibilities at the present time. However, with the ability to generate specific deletions and translocations by using "chromosome engineering" strategies (17), it may be possible to generate chromosomes with DRT/DMC with defined deletion or translocation breakpoints that will allow us to distinguish between these possibilities.

Genetic Instability. Genetic instability occurs in cancer cells at distinct levels. In most cancers, the instability occurs at the chromosome level, resulting in gains or losses of whole chromosomes or large portions of chromosomes (18). Marker chromosomes, containing complicated rearrangements involving more than one chromosome, are quite common in solid tumors (19). The molecular basis for the multiple rearrangements that occur during the generation of marker chromosomes is not understood. However, the results presented here indicate that certain chromosome translocations are unstable and undergo numerous secondary rearrangements.

Another example of chromosomal instability occurs at chromosomal fragile sites. Chromosomal fragile sites are loci found on chromosomes that are susceptible to forming gaps, breaks, or rearrangements in metaphase chromosomes when cells are cultured under conditions that delay DNA replication (20). Furthermore, chromosomal fragile sites are late replicating (21). One attractive model to explain the unstable nature of chromosomal fragile sites is that incomplete chromatin condensation caused by late replication and/or incomplete replication itself leads to gaps and breaks at these sites during the subsequent mitosis (22). Given the unstable nature and late replication phenotype observed with the chromosome rearrangements described here, we propose that *t*ranslocation *in*stability (TIN) arises due to incomplete chromosome condensation and/or incomplete replication that persists into mitosis. A model to explain the numerous rearrangements that occur on chromosomes with DMC/DRT is shown in Fig. 6*B*.

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